REMARKS

Claims 1, 3-6, and 8-35 were pending at the time of the Office action. Claims 33 and 34 have been withdrawn from consideration. Claims 1, 3, 4, 6, 8-20, and 29 stand rejected under 35 U.S.C. § 102. Claims 1, 3-6, 8-32, and 35 stand rejected under 35 U.S.C. § 103. Applicant addresses these rejections as follows.

Amendment to the Specification

The Examiner has objected to the language of the Abstract. In response, Applicant has amended the Abstract, as suggested by the Examiner. This objection should now be withdrawn.

Amendments to the Claims

Claim 35 has been amended to reflect the amendment to claim 1. New claim 36 has been added. Support for claim 36 is found, for example, in claim 35 and in the specification at Examples 1 and 2.

Applicant reserves the right to pursue any cancelled subject matter in this or in a continuing application. No new matter has been added.

Rejections under 35 U.S.C. § 102

Claims 1, 3, 4, 6, 8-20, and 29 stand rejected under 35 U.S.C. § 102(b) as being anticipated by Bickers et al. (*The Journal of Dermatology* 27: 691-695, 2000; herein "Bickers"), as evidenced by Dou et al. (U.S. Patent Application Publication No. 2002/0151582; herein "Dou"). The Examiner states (Office action, page 3) that Bickers "teach[es] that... green tea, black tea and constituent polyphenols protect against chemical- and ultraviolet B (UVB)-induced carcinogenesis and reduce the growth of established tumors in skin" and cites Dou as evidentiary support that the green tea

extracts of Bickers contain polyphenolic compounds (e.g., EGCG). This rejection is respectfully traversed.

The present claims are directed to a method of treating precancerous lesions (e.g., actinic keratoses) by administration of a polyphenol-containing composition to a patient. Applicant has demonstrated the effectiveness of this method and describes these results in Examples 1 and 2 of the specification as filed. As described, patients with precancerous lesions were treated in a clinical setting with a polyphenol-containing composition, and this treatment resulted in complete elimination of the precancerous lesions.

Bickers does not anticipate the present claims because this reference fails to teach a method for treating a precancerous lesion of the skin by administering a pharmaceutically effective amount of a polyphenol to a patient, as required by claim 1. In contrast to the claimed methods, Bickers instead teaches the prevention of a precancerous lesion, a result far different from treatment of an already established precancerous condition. This distinction is first highlighted by the title of Bickers, "Novel Approaches to Chemoprevention of Skin Cancer" (emphasis added). Moreover, throughout Bickers, prevention or protection against precancerous lesions is repeatedly discussed. For example, Bickers states:

> Protection against sun-induced damage leading to photocarcinogenesis in skin is a highly desirable goal (Abstract).
>
> [T]wo major polyphenolic sub-fractions protect against UVB-induced erythema in SKH-1 mice... and topical application of tea extracts to human volunteers protects against UVB-induced erythema (Abstract).
>
> These studies verify a conceptual rationale for employing naturally occurring dietary constituents as an approach to cancer chemoprevention (Abstract).
>
> [K]nown dietary agents with chemopreventive activity are of great potential interest... (page 692, left column of Bickers, under "Introduction").

> Our laboratory has had a long-standing interest in cancer chemopreventive potential of tea extracts (page 692, left column of Bickers, under "Introduction").
>
> Recently, green tea was also shown to afford protection against PUVA-induced early damage in skin. (page 693, left column of Bickers, under "Anti-Inflammatory Activity of Tea Extracts").

Turning specifically to the passages cited by the Office, Applicant notes that each of these teachings similarly refers to either the protective effect of standardized green tea extract or the treatment of cancerous tumors. The Examiner first focuses on a passage (Office action, page 3 and Abstract of Bickers) that states "green tea, black tea and constituent polyphenols protect against chemical- and ultraviolet B (UVB)-induced carcinogenesis and reduce the growth of established tumors in skin" (emphasis added). This is consistent with Applicant's characterization of the reference as failing to treat precancerous skin lesions.

The Examiner further notes that "standardized green tea extract (SGTE) prior to and during treatment of SKH-1 mice diminished PUVA-induced skin hyperplasia and hyperkeratosis (precancerous sin (*sic*) lesion)" (Office action, page 3 and Abstract of Bickers). This statement is based on the result in Bickers that "oral administration of green tea extract prior to or during multiple PUVA treatments of SKH-1 hairless mice reduces hyperplasia, hyperkeratosis, erythema and edema" (page 693, right column of Bickers, under "Anti-Inflammatory Activity of Tea Extracts"). Again, the green tea extract is preventing PUVA damage, and not treating an established lesion. As noted specifically by Bickers when describing these results, the administration of a green tea extract prior to PUVA treatment or during PUVA treatment provides only a protective effect against such conditions (page 693, left column of Bickers, under "Anti-Inflammatory Activity of Tea Extracts"). Indeed, in none of the above passages does Bickers indicate that the extracts provide a therapeutic effect.

The Examiner also notes (Office action, page 3) that Bickers teaches that "[t]opical application of SGTE to human skin prior to PUVA-treatment inhibited the delayed skin inflammatory response" (emphasis added). This statement, however, again refers to a protective effect. The same is true of the citation (Office action, page 3) that "oral and topical administration of standardized black tea extract and its two major polyphenolic sub-fractions protect against UVB-induced erythema in SKH-1 mice" (emphasis added).

Furthermore, the Office cites experimental studies that have shown the efficacy of tea constituents as inhibitors of carcinogenesis-associated surrogate markers or inflammation (page 4 and page 693, right column of Bickers, under Anticarcinogenic Effects of Tea Extracts in Humans"). As reported by Bickers, pretreatment of the back skin of normal human volunteers protected against UVB (2 MED)-mediated induction of sunburn-cell formation and depletion of CD1a+ Langerhans cells. Bickers concludes that "topically applied green tea is effective in abrogating PUVA-induced inflammatory responses in human skin" (page 693, right column of Bickers, under "Anticarcinogenic Effects of Tea Extracts in Humans"). This statement again fails to address treatment of established precancerous lesions, relating instead to a protective effect of the green tea extract against UV damage.

Finally, Applicant points to a statement made in Bickers and cited by the Examiner, that "both black and green tea and their constituents inhibit the tumor initiation, promotion and malignant progression stages of multi-step skin carcinogenesis" (page 693, left column of Bickers, under "Anticarcinogenic Effects of Tea Extracts in Animal Models"). This passage of Bickers cites Mukhtar et al. (*Toxicological Sciences* 52: 111-117, 1999), provided herewith, as describing the source of this result. But, as in Bickers, Mukhtar describes only chemoprevention of cancer by green tea, and not the treatment of a precancerous lesion. Specifically, Mukhtar states:

> [W]e showed that EGCG induces apoptosis and cell cycle arrest in human epidermoid carcinoma (A431) cells (Ahmad *et al.*, 1997). Importantly, this apoptotic response was specific for

> cancer cells, since EGCG treatment also resulted in the induction of apoptosis in human carcinoma keratinocytes HaCaT, human prostate carcinoma cells DU145, and mouse lymphoma cells LY-R, but not for normal human epidermal keratinocytes. (Page 113, right column of Mukhtar; emphasis added.)

Thus, as described in Mukhtar (citing Ahmad et al. (*Journal of the National Cancer Institute* 89: 1881-1886, 1997, provided herewith), treatment with epigallocatechin-3-gallate stimulated apoptosis of HaCaT, L5178Y, and DU145 cells, but not of normal human epidermal keratinocytes (see, e.g., the abstract of Ahmad). Thus, Mukhtar and Ahmad, the references cited by Bickers, clarify that the statement relied upon by the Office relates only to cancerous cells, and teaches nothing about the effects of the administration of a polyphenol on precancerous cells (e.g., the skin cells of claim 1). The other reference cited by the Examiner, Dou, does not relate to treatment of precancerous lesions. Therefore, Bickers, alone or as evidenced by Dou, fails to teach all the elements of claims 1, 3, 4, 6, 8-20, and 29, and Applicant respectfully requests that this rejection of the claims under 35 U.S.C. § 102 be withdrawn.

Rejections under 35 U.S.C. § 103

Claims 1, 3-6, 8-26, and 29 stand rejected under 35 U.S.C. § 103(a) as being unpatentable over Bickers, as evidenced by Dou. This rejection is respectfully traversed. Bickers, as evidenced by Dou, fails to support a *prima facie* case of obviousness. As described above, the Bickers teaching relates only to the prevention of a precancerous lesion or the effect of green tea extract on cancerous cells. Indeed, Mukhtar and Ahmad teach that EGCG has no effect on human epidermal keratinocytes and therefore, if anything, teach away from the present invention. Applicant respectfully requests that this rejection of the claims under 35 U.S.C. § 103(a) be withdrawn.

Claims 1, 3-6, 8-32, and 35 stand further rejected under 35 U.S.C. § 103(a) as being unpatentable over Bickers in view of Brash et al. (U.S. Patent Application

Publication No. 2002/0198161; herein "Brash") and further in view of Voet (U.S. Patent No. 6,723,750; herein "Voet") as evidenced by Dou. Brash and Voet fail to cure the deficiencies of Bickers, as neither indicates that polyphenol-containing compounds treat precancerous lesions. Reconsideration and withdrawal of this second bases for the rejection is also respectfully requested.

CONCLUSION

Applicant submits that the claims are now in condition for allowance, and such action is respectfully requested.

Enclosed is a Petition to extend the period for replying to the Office action for one month, to and including October 17, 2008, and a check in payment of the required extension fee. If there are any charges or any credits, please apply them to Deposit Account No. 03-2095.

Respectfully submitted,

Date: 17 October 2008

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Green Tea in Chemoprevention of Cancer

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The concept of prevention of cancer using naturally occurring substances that could be included in the diet consumed by the human population is gaining increasing attention. Tea, next to water, is the most popularly consumed beverage in the world and it is grown in about 30 countries. Abundant data, amassed from several laboratories around the world in the last ten years, provided convincing evidence that polyphenolic antioxidants present in tea afford protection against cancer risk in many animal-tumor bioassay systems. The epidemiological studies, though inconclusive, have also suggested that the consumption of tea is associated with a lowered risk of cancer. Much of this work has been done on green tea; less is known about black tea. Green tea contains many polyphenolic antioxidants, and (–)-epigallocatechin-3-gallate (EGCG) is the key polyphenolic antioxidant believed to be responsible for most of the cancer chemopreventive properties of green tea. This review will discuss these effects and the molecular mechanisms associated with the biological response to green-tea polyphenols.

Key Words: green-tea polyphenols; epigallocatechin-3-gallate (EGCG); cancer; chemoprevention.

Chemoprevention has emerged as a practical approach to reducing cancer incidence and therefore the mortality and morbidity associated with it. At present, at least 30 different groups of agents are known, from laboratory studies, to have cancer chemopreventive properties. Some of these agents are also showing promise in epidemiological studies (Challa *et al.*, 1997; Chung *et al.*, 1998; Dragsted *et al.*, 1993; Kelloff *et al.*, 1996; Pezzuto, 1997). One such group of agents is known as "polyphenols." Tea, next to water, is the most popular beverage consumed by humans, and it contains polyphenolic constituents known as "catechins" (Ahmad *et al.*, 1998; Katiyar and Mukhtar 1996). The anti-carcinogenic and anti-mutagenic activity of polyphenolic agents present in green tea were first reported almost a decade ago (Khan *et al.*, 1988, Wang *et al.*, 1989a,b). Green tea is derived from *Camellia sinensis,* an evergreen shrub of the *Theaceae* family. Tea, for many generations, has been considered to possess health-promoting potential in some parts of the world (Weisburger *et al.*, 1997). Epidemiological studies, though inconclusive, suggest that the consumption of green tea is associated with a lower risk of cancer. The majority of studies assessing the usefulness of tea in prevention of cancer have been conducted with green tea, whereas in a few studies, the chemopreventive potential of black tea has also been assessed (Katiyar and Mukhtar, 1996; Ahmad *et al.*, 1998). Based on studies in cell culture, laboratory animals, and epidemiological observations, clinical trials of green tea consumption and cancer risk have been initiated. The major polyphenolic antioxidants that are thought to be responsible for the cancer chemopreventive potential of green tea include (–)-epicatechin (EC), (–)-epigallocatechin (EGC), (–)-epicatechin-3-gallate (ECG), and (–)-epigallocatechin-3-gallate (EGCG). Among these, EGCG is believed to be the most protective agent (Ahmad *et al.*, 1998; Katiyar and Mukhtar 1996).

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History of Tea Consumption

The plant *Camellia sinensis* was originally discovered and grown in Southeast Asia thousands of years ago, and according to the Chinese mythology, the emperor Shen Nung discovered tea for the first time in 2737 BC (Harbowy and Balentine, 1997). Since then, the popularity of this beverage has increased to a point where, at present is, next only to water, the most popular beverage around the world. The per capita worldwide consumption is approximately 120 ml brewed tea per day (Ahmad *et al.*, 1998; Katiyar and Mukhtar 1996). Tea is currently grown and cultivated in at least 30 countries around the world (Ahmad *et al.*, 1998; Katiyar and Mukhtar 1996). Many types of tea preparations, originating from the same plant source (*Camellia sinensis*) but having different processing methods, are consumed today. The three most popular major tea types include black tea (78%, mainly consumed in Western and some Asian countries), green tea (20%, mainly consumed in Asia, and a few countries in North Africa and the Middle East), and oolong tea (2%, consumed in some parts of China and Taiwan) (Ahmad *et al.*, 1998; Katiyar and Mukhtar 1996).

Tea and Cancer Chemoprevention

A number of studies in laboratory animals in various target-organ bioassay protocols, conducted in many laboratories around the world, have provided convincing evidence that the polyphenolic antioxidants present in tea are capable of affording protection against cancer initiation and its subsequent development (Ahmad *et al.*, 1998; Katiyar and Mukhtar 1996). Data from various epidemiological studies conducted in dif-

ferent populations, though inconclusive, were considered to be of sufficient merit to embark on clinical trials evaluating the association of green tea consumption with cancer risk (Ahmad *et al.*, 1998; Katiyar and Mukhtar 1996; Kohlmeier *et al.*, 1997).

Oral consumption or topical applications of green tea and/or its polyphenolic constituents have been show to afford protection against chemical carcinogen- as well as ultraviolet radiation- induced skin carcinogenesis in the mouse model (Mukhtar *et al*, 1994). In many other animal studies, the polyphenolic fraction-isolated green tea, the water extract of green tea, or individual polyphenolic antioxidants present in green tea have also been shown to afford protection against chemically induced carcinogenesis in lung, liver, esophagus, forestomach, duodenum, pancreas, colon, and breast (Ahmad *et al.*, 1998; Katiyar and Mukhtar 1996). Based on recent studies, it is now believed that much of the cancer chemopreventive properties of green tea are mediated by (–)-epigallocatechin-3-gallate (EGCG) (Ahmad *et al.*, 1998; Katiyar and Mukhtar 1996). It is appreciated that other polyphenolic agents present in green tea also contribute to its cancer chemopreventive efficacy. However, it is not clear whether all the polyphenolic compounds of green tea work through a similar biochemical pathway or by different mechanisms.

An ideal chemopreventive agent for human use should have little or no toxicity, high efficacy in multiple sites, capability of oral consumption, a known mechanism of action, low cost, and human acceptance. A single cup of brewed green tea contains up to 400 mg of polyphenolic antioxidants, of which 200 mg is EGCG. It is also interesting to observe that at present, many available consumer products such as drinks, ice creams, healthcare products, and cosmetics are supplemented with green tea extracts.

Bioavailability of Tea Polyphenols

The bioavailability of the active polyphenolic constituents, after tea consumption by laboratory animals and humans, is poorly defined. Yang *et al* (1998) conducted a study in 18 individuals who were given different amounts of green tea, and plasma concentrations and urinary excretion of tea catechins were measured as a function of time. After consuming 1.5, 3.0, or 4.5 g of decaffeinated green tea (in 500 ml of water), the maximum plasma concentrations (C_{max}) of EGCG, EGC, and EC were found to be 326 ng/ml, 550 ng/ml, and 190 ng/ml respectively, as observed at 1.4–2.4 h after ingestion of the tea preparation. When the dose was increased from 1.5 to 3.0 g, the C_{max} values were found to increase by 2.7–3.4-fold, but further increasing the dose to 4.5 g did not increase the C_{max} values significantly, suggesting a saturation phenomenon. At all the concentrations employed, the half-life of EGCG was found to be longer than the half-life of EGC or EC. EGC and EC, but not EGCG were excreted in urine, and it was demonstrated that 90% of the total urinary EGC and EC were excreted within 8 h. When the tea dosage was increased, the amount of EGC and EC excretion also increased, but a clear dose-response relationship was not observed. This study provided basic pharmacokinetic parameters of green tea polyphenols in humans, which may be used to estimate the levels of these compounds after drinking green tea.

Recently, Saganuma *et al* (1998) studied the distribution of radiolabeled [^{3}H]EGCG in mouse organs following oral administration. Radioactivity was found in many organs, including those where inhibition of carcinogenesis by EGCG or green tea extract has already been shown. These results suggested that frequent consumption of green tea enables the body to maintain a high level of tea polyphenols. These studies may be useful in designing future strategies aiming towards the development of green tea as a practical chemopreventive agent.

Mechanism(s) of Biological Effects of Green Tea

Initial Mechanistic Studies

A proper understanding of mechanisms of the biological effects imparted by green tea is essential, as it may be helpful in designing and improving the strategies for cancer chemoprevention. The initial mechanistic attempts in this direction were largely focused on assessing the effect of green tea polyphenols on the following:

- prevention against mutagenicity and genotoxicity of chemicals
- reduction in biochemical markers of tumor initiation
- reduction in biochemical markers of tumor promotion
- regulation of detoxification enzymes
- trapping of activated metabolites of carcinogens
- regulation of antioxidant and free-radical scavenging activity (Katiyar and Mukhtar, 1996).

Molecular Mechanisms

A summary of the research work conducted to decipher the molecular mechanisms involved with the biological responses of green tea polyphenols is given below.

Green tea polyphenols activate the mitogen-activated protein kinase (MAPK) pathway. The protective effects of green tea polyphenols have been attributed to the inhibition of enzymes such as the cytochromes P450, which are involved in the bio-activation of carcinogens (Yu *et al.*, 1997). Some other *in vivo* studies have also demonstrated the involvement of Phase II detoxification enzymes during the biological response to green tea. Because the 5′ flanking regions of Phase II genes contain an antioxidant-responsive element (ARE) which is believed to mediate the induction of Phase II enzymes by many drugs, the involvement of the MAPK pathway was studied as a mechanism of biological response to green tea polyphenols. This study demonstrated that the activation of the MAPK pathway might be due to a potential signaling pathway involved in the regulation of ARE-mediated phase II enzyme

gene expression (Yu *et al.*, 1997). It was also demonstrated that green tea polyphenol treatment of human hepatoma (HepG2) cells transfected with a plasmid construct containing ARE and a minimal glutathione S-transferase Ya promoter linked to the chloramphenicol acetyltransferase (CAT) reporter gene causes an induction of CAT activity. This result suggests that green tea polyphenols stimulate the transcription of Phase II detoxifying enzymes through ARE. Green tea polyphenol treatment also resulted in a significant activation of MAPK, extracellular, signal-regulated kinase 2 (ERK2), as well as c-*Jun* N-terminal kinase 1 (JNK1) and an increase in the mRNA levels of early response genes c-*jun* and c-*fos*.

EGCG inhibits urokinase activity. Recently, it was proposed that the anti-cancer activity of EGCG is associated with the inhibition of urokinase, which is one of the most frequently expressed enzymes in human cancers (Jankun *et al.*, 1997). Through the use of computer-based molecular modeling, it was demonstrated that EGCG binds to urokinase, blocking the histidine 57 and serine 195 of urokinase catalytic triad and extending towards arginine 35 from a positively charged loop of urokinase. These calculations were verified by assessing the inhibition of urokinase activity by the spectrophotometric amidolytic assay. However, the practicability of this study with achievable dose levels was later challenged by Yang (1997).

Green tea induces apoptotic cell death and arrest of the cell cycle. Because the life span of both normal and cancer cells within a living system is significantly affected by the rate of apoptosis (Fesus *et al.*, 1995), chemopreventive agents that could modulate apoptosis might affect the steady-state cell population. On one hand, several cancer chemopreventive agents induce apoptosis, but on the other hand, the tumor-promoting agents are found to inhibit apoptosis (Boolbol *et al.*, 1996; Mills *et al.*, 1995; Wright *et al.*, 1994). Therefore, it can be assumed that the chemopreventive agents with proven effects in animal tumor bioassay systems and/or human epidemiology, and the ability to induce apoptosis of cancer cells, may have wider implications for the management of cancer. At present, only a limited number of chemopreventive agents are known to cause apoptosis (Jee *et al.*, 1998; Jiang *et al.*, 1996). In our laboratory, we showed that EGCG induces apoptosis and cell cycle arrest in human epidermoid carcinoma (A431) cells (Ahmad *et al.*, 1997). Importantly, this apoptotic response was specific for cancer cells, since EGCG treatment also resulted in the induction of apoptosis in human carcinoma keratinocytes HaCaT, human prostate carcinoma cells DU145, and mouse lymphoma cells LY-R, but not for normal human epidermal keratinocytes.

Another study (Chen *et al.*, 1998) compared the effect of EGCG on the growth of SV40 virally transformed human fibroblasts (WI38VA) with that of normal WI38 cells. In this study, EGCG was found to inhibit the growth of the transformed WI38VA cells, but not of their normal counterparts. This study further demonstrated a similar differential growth inhibitory effect of EGCG between human colorectal cancer (Caco-2) cells, breast cancer (Hs578T) cells, and their respective normal counterparts. EGCG treatment also induced apoptosis, and enhanced serum-induced expression of c-*fos* and c-*myc* genes in transformed WI38VA cells, but not in the normal WI38 cells. This study suggested that the differential modulation of certain genes such as c-*fos* and c-*myc*, could be responsible for these differential responses of EGCG.

In another study (Fujiki *et al.*, 1998), it was demonstrated that EGCG and other tea polyphenols inhibit growth of human lung cancer (PC-9) cells with a G2/M phase arrest of the cell cycle. This study demonstrated that [^{3}H]EGCG, administered by po intubation into the mouse stomach, results in small amounts of ^{3}H-activity in many organs such as skin, stomach, duodenum, colon, liver, lung, and pancreas, where EGCG and green tea extract have been shown to have anticarcinogenic effects. In this study, the involvement of the tumor necrosis factor (TNF)-α pathway was suggested as a mechanism of EGCG-mediated biological responses.

In another study by Yang *et al* (1998), the growth inhibitory effects of green tea polyphenols were investigated using 4 human cancer cell lines. Growth inhibition was measured by ^{3}H-thymidine incorporation after 48 h of treatment. EGCG and EGC displayed strong growth inhibitory effects against lung-tumor cell lines H661 and H1299, with estimated IC_{50} values of 22 μM, but were less effective against lung-cancer cell line H441 and colon-cancer cell line HT-29 with IC_{50} values 2- to 3-fold higher. ECG was found to have lower activities whereas EC was even less effective. In this study, exposure of H661 cells to a dose of 30 μM EGCG, EGC, or theaflavins for 24 h resulted in a dose-dependent apoptosis. The incubation of H661 cells with EGCG also resulted in a dose-dependent formation of H_2O_2. Addition of H_2O_2 to H661 cells resulted in an apoptotic response similar to EGCG. EGCG-induced apoptosis in H661 cells was found to be completely inhibited by exogenously added catalase (50 units/ml). This inhibition suggests that tea polyphenol-mediated H_2O_2 production results in apoptosis of the cells, contributing to the growth inhibitory potential of tea polyphenols *in vitro*. In this study, the involvement of H_2O_2, as well as the effect of catalase, was an intriguing observation because the tea polyphenols are generally regarded as antioxidants. The explanation provided by the authors is that tea polyphenols also possess pro-oxidative activities (Yang *et al.*, 1998).

EGCG inhibits cellular proliferation and tumor progression via epidermal growth factor receptor (EGFR) pathway. The activation of the epidermal growth factor receptor (EGFR) tyrosine kinase by its ligand is believed to initiate multiple cellular reponses associated with cell proliferation on one hand. The over-expression of EGFR is shown to produce neoplastic phenotype on the other hand. Based on these facts, a recent study by Liang *et al.* (1997) demonstrated that EGCG significantly inhibits both DNA synthesis and the protein ty-

rosine kinase activities of EGFR, platelet-derived growth-factor receptor (PDGFR), and fibroblast growth-factor receptor (FGFR), but not of $pp60^{v\text{-}src}$, protein kinase C (PKC), or protein kinase A (PKA), in A431 cells. EGCG was also found to inhibit the auto-phosphorylation of EGFR by EGF and to block the binding of EGF to its receptor. This study suggested that EGCG might inhibit tumor development by blocking the EGFR-pathway.

EGCG inhibits the induction of nitric oxide (NO)-synthase via a down-regulation in the transcription factor nuclear factor-κB (NFκB). Nitric oxide (NO) is a bioactive molecule that plays an important role in inflammation and carcinogenesis, and in a recent study, Lin and Lin (1997) assessed the effects of green tea polyphenols on the modulation of NO-synthase in thioglycollate-elicited and lipopolysaccharide (LPS)-activated peritoneal macrophages. Gallic acid (GA), EGC, and EGCG were found to inhibit the protein expression of inducible NO-synthase as well as the generation of NO. This study further demonstrated that EGCG inhibits the activation of the transcription factor NFκB, an event that is believed to be associated with the induction of inducible NO-synthase (iNOS). Taken together, these data suggested that EGCG may block the early event of NO-synthase induction via inhibiting the binding of transcription NFκB to the iNOS promoter, thereby, inhibiting the induction of iNOS transcription. The theory of involvement of NO in the biological response of EGCG was strengthened by another study by Chan et al. (1997), who demonstrated that EGCG causes an inhibition of lipopolysaccharide (LPS)- and interferon (IFN)-γ-activated iNOS mRNA expression in a cell-culture system. EGCG was also found to inhibit the enzyme activities of iNOS and neuronal NO-synthase (nNOS).

Peroxynitrite (OONO) is a highly toxic oxidizing and nitrating species that is produced *in vivo* via a reaction between superoxide radical (O^{2}-) and NO. In another study, Pannala *et al* (1997) demonstrated the ability of green tea polyphenols, *viz.*, catechin, epicatechin, ECG, EGC, and EGCG to (i) inhibit OONO-mediated tyrosine-nitration, and (ii) limit surface charge alteration of low density lipoprotein (LDL). In this study, all the compounds tested were found to be potent OONO scavengers, as they were effective in preventing the nitration of tyrosine. These polyphenols were also found to protect against OONO-mediated LDL modification.

EGCG inhibits tumor promoter-mediated activator protein-1 (AP-1) activation, and cell transformation. Because many studies have suggested recently that the activation of AP-1 plays an important role in tumor promotion, the down-regulation of this transcription factor is now thought to be a general therapeutic strategy against cancer (McCarty, 1998). In a recent study (Dong *et al.*, 1997) employing the JB6 mouse epidermal cell line, an extensively used in vitro model system for tumor promotion studies, Dong et al investigated anti-tumor promoting effects of EGCG and theaflavins. Both of these were found to inhibit EGF- or TPA- induced cell transformation, as well as AP-1-dependent transcriptional activity and DNA binding activity. This study further showed that the inhibition of AP-1 activation occurs via the inhibition of a c-Jun NH2-terminal kinase (JNK)-dependent pathway.

EGC inhibits the activity of the protein tyrosine kinase, c-jun mRNA expression, and JNK1 activation. In a recent study, Lu *et al* (1998) investigated some possible mechanisms involved with the antiproliferative ability of EGC. Employing rat aortic smooth-muscle (A7r5) cells, it was demonstrated that the activity of the serum-stimulated membranous protein, tyrosine kinase (PTK), is inhibited by EGC. EGC was also found to reduce the phosphorylation of many proteins with different molecular weights, at the tyrosine site, indicating that EGC may inhibit activity of tyrosine kinase, or stimulate the activity of phosphatase. It was further demonstrated that EGC reduces the levels of c-*jun* mRNA, phosphorylated JNK1, and JNK1-kinase activity. These data suggest that the antiproliferative effect of EGC, at least in part, is mediated through the inhibition of tyrosine kinase activity, reducing c-*jun* mRNA expression and inhibiting JNK1 activation.

The involvement of PTK activity and protein phosphorylation was further explained by another study, where Kennedy *et al* (1998) evaluated the mechanism of the antiproliferative potential of green tea polyphenols in Ehrlich ascite tumor cells. In this study, EGC and EGCG treatments were found to result in a significant decrease in cell viability. EGC, but not EGCG, caused a stimulation of PTK activity. EGC treatment was also found to result in tyrosine phosphorylations of 42 and 45 kDa proteins, and in the activity of ornithine decarboxylase (ODC), an essential cellular enzyme in polyamine biosynthesis.

Skin Effects of Green Tea

Skin is the largest body organ and serves as a protective barrier against the deleterious effects of environmental insults, including those caused by ultraviolet (UV) radiation. Much of the deleterious effect of solar UV radiation is because of UVB (290–320 nm). Although the long-term abnormalities of UVB typically become evident in the population aged 50 years and beyond, epidemiological studies indicate that much of the critical sunlight exposure responsible for these adverse effects is received at a young age. Recent epidemiological observations suggest that individuals with a history of non-melanoma skin cancer have increased risk of melanoma and certain non-cutaneous cancers.

UVB induces skin cells to produce reactive oxygen species, eicosanoids, proteinases, and cytokines, and inhibition of these mediators is thought to reduce skin damage. Evidence for this comes from the demonstration that antioxidants such as ascorbic acid and alpha tocopherol produce photoprotective effects in some *in vitro* and *in vivo* studies (Elmets and Mukhtar, 1996; Mukhtar and Elmets, 1996).

Studies have suggested that green tea polyphenols may be

useful in affording protection against inflammatory responses and against skin-cancer risk. Topical application of green tea polyphenols to mouse skin inhibits 12–0-tetradecanoylphorbol-13-acetate and other skin tumor-promoter-caused induction of protein and mRNA expression of the pro-inflammatory cytokines interleukin (IL)-1 α and TNF-α. Skin application of green tea polyphenols inhibits UV-radiation-induced local and systemic suppression of contact hypersensitivity and edema responses in C3H/HeN mice. In many *in vitro* studies, green tea polyphenols or crude extracts of green tea have also shown preventive effects in systems considered essential in inflammatory and carcinogenic processes (Ahmad *et al.*, 1997; Katiyar and Mukhtar, 1996).

The relevance of the extensive *in vitro* and *in vivo* laboratory data on adverse effects caused by solar UVB in human skin is not clear. This information can be derived either based on epidemiological studies in a high-risk population, or based on using short-term assays with noninvasive techniques and acceptable protocols in human volunteers. Recently, we assessed the protective effect on skin of the application of green tea polyphenols against UV-induced erythema in human volunteers. In this study, a polyphenolic fraction obtained from green tea was applied, in different strengths, on the untanned backs of normal volunteers. Thirty min later the sites were exposed to twice the minimal erythemogenic dose (MED) of UV radiation from a solar simulator. Sites pretreated with green tea polyphenols exhibited significantly less erythema when compared to vehicle-treated sites. The photoprotective effects of green tea polyphenols were dependent on the strength of the dose applied, with maximum protection observed from 200 μl of a 5% solution. In time course studies, the green tea polyphenol-mediated cutaneous photoprotective effect was evident, even when UV irradiation was delayed for many h. The protective effects lasted for at least 72 h, thus indicating a relatively long-term protection, particularly against chronic low-dose environmental insult. Skin application of green tea polyphenols to human volunteers also resulted in significant protection against 2 MED-induced enhancement of sunburn-cell formation and depletion of CD1 a^+ Langerhans cell density (Mukhtar *et al.*, 1996).

In additional studies, we investigated (Katiyar *et al.*, 1999) whether or not the topical application of EGCG would protect against UVB-induced adverse effects in human skin. In this study, we assessed the effect of EGCG treatment on inhibition of UVB-induced infiltration of leukocytes (macrophage/neutrophils), a potential source of generation of ROS and prostaglandin (PG) metabolites, which play a critical role in skin-tumor promotion in multistage skin carcinogenesis. Human subjects were exposed to UVB radiation (at 4 MED doses) on sun-protected skin, and skin biopsies or keratomes were obtained 24 h or 48 h later. We found that topical application of EGCG (3 mg/2.5 cm^2) before UVB exposure to human skin significantly blocked UVB-induced infiltration of leukocytes and reduced myeloperoxidase activity. The infiltration of leukocytes is considered the major source of generation of ROS. In the same set of experiments, we found that the topical application of EGCG before UVB exposure decreased UVB-induced erythema. In additional experiments, we found that EGCG treatment before UVB exposure produced significantly lower PG metabolites, particularly PGE_2, in the epidermal microsomal fraction of the skin, as compared to non-pretreated skin. Histological examination of skin revealed that EGCG-pretreated and UVB-exposed human skin contained fewer dead cells in the epidermis when compared to non-pretreated, UVB-exposed skin. These data demonstrate that EGCG has the potential to block the UVB-induced infiltration of leukocytes and the subsequent generation of ROS in human skin. This may be responsible, at least in part, for the anti-inflammatory effects of green tea.

Based on the work described above, it is tempting to suggest that the use of GTP in cosmetic preparations may be a novel approach for preventing the adverse effects associated with UV radiation in humans. It is of interest that many low-priced cosmetics marketed by small companies, as well as expensive lines of cosmetics marketed by the name brand companies, are supplementing their products with green tea extracts.

Modulatory Effects of Green Tea for Cancer Chemotherapy

A recent study (Sadzuka *et al.*, 1998) has shown that green tea can also modulate the efficacy of cancer-chemotherapeutic drugs in such a way that it increases the drugs' efficacy. In this study, the oral administration of green tea enhanced the tumor-inhibitory effects of doxorubicin on Ehrlich ascite carcinomas implanted in CDF_1 and BDF_1 mice. Green tea treatment resulted in an increased availability of doxorubicin in tumor, but not in normal tissue. If verified in the human population, these observations may have relevance to cancer chemotherapy.

Conclusion and Future Directions

Since 1990, approximately ten million new cases of cancer were diagnosed and four million cancer-related deaths have occurred. It is believed that almost one third of the cancers are caused by dietary habits and the manipulation in diet is increasingly being recognized as a potential strategy against cancer (Katiyar and Mukhtar, 1996; Weisburger, 1996). The use of tea, especially green tea, as a cancer chemopreventive agent has only been appreciated in the last ten years. Green tea is a popularly consumed beverage, is relatively inexpensive and non-toxic, and has been shown to afford protection against many cancer types. The epidemiological as well as laboratory studies have shown an inverse association of green tea consumption with the development of certain cancer types. Although compelling evidence is now available that shows the preventive potential of green tea against cancer, a clear understanding of the mechanisms associated with its action is far from complete. A complete knowledge of the molecular mechanism(s) involved with the anti-carcinogenic efficacy of green

tea polyphenols may be useful in devising better chemopreventive strategies against cancer.

In view of the available data from laboratory and epidemiological studies, clinical trials are now warranted to evaluate the usefulness of green tea and the polyphenolic antioxidants present therein. Vastag (1998) realized that a cup of tea provides an antioxidant boost that may protect against several cancer types, and that these tea antioxidants are much more potent than vitamins C and E in their ability to scavenge potentially carcinogenic free radicals. It is important to emphasize here that Phase I Clinical Trials to evaluate the possible efficacy of formulated green tea, in patients with advanced solid tumors, are currently underway at M.D. Anderson Cancer Center and Memorial Sloan-Kettering Cancer Center.

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Green Tea Constituent Epigallocatechin-3-Gallate and Induction of Apoptosis and Cell Cycle Arrest in Human Carcinoma Cells

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Background and Purpose: **The polyphenolic compounds present in green tea show cancer chemopreventive effects in many animal tumor models. Epidemiologic studies have also suggested that green tea consumption might be effective in the prevention of certain human cancers. We investigated the effect of green tea polyphenols and the major constituent, epigallocatechin-3-gallate, on the induction of apoptosis (programmed cell death) and regulation of cell cycle in human and mouse carcinoma cells.** ***Methods:*** **Human epidermoid carcinoma cells (cell line A431), human carcinoma keratinocyte (cell line HaCaT), human prostate carcinoma cells (cell line DU145), mouse lymphoma cells (cell line L5178Y), and normal human epidermal keratinocytes (NHEKs) were used. Apoptosis was assessed by 1) the formation of internucleosomal DNA fragments by agarose gel electrophoresis, 2) confocal microscopy, and 3) flow cytometry after tagging the DNA fragments by fluorescence label. The distribution of cells in different phases of the cell cycle was analyzed by flow cytometry.** ***Results:*** **Treatment of A431 cells with green tea polyphenols and its components, epigallocatechin-3-gallate, epigallocatechin, and epicatechin-3-gallate, resulted in the formation of internucleosomal DNA fragments, characteristic of apoptosis. Treatment with epigallocatechin-3-gallate also resulted in apoptosis in HaCaT, L5178Y, and DU145 cells, but not in NHEK. Confocal microscopy and flow cytometry confirmed the findings. The DNA cell cycle analysis showed that in A431 cells, epigallocatechin-3-gallate treatment resulted in arrest in the G_0–G_1 phase of the cell cycle and a dose-dependent apoptosis.** ***Conclusions:*** **Green tea may protect against cancer by causing cell cycle arrest and inducing apoptosis. It needs to be evaluated in human trials.**

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The term "Chemoprevention" is coined for cancer prevention and cancer control by use of naturally occurring and/or synthetic compounds. The occurrence of the disease may be completely prevented, blocked, or reversed. This approach is promising because chemotherapy and surgery have not been fully effective against the high incidence or low survival rate of most of the cancers *(1)*. In this regard, the naturally occurring antioxidants present in the diet and beverages consumed by humans are receiving increasing attention *(2)*.

Tea consumption in the world is very high and ranks second to water consumption. It is prepared from the dried leaves of *Camellia sinensis*. Most tea consumed in the world can be classified in two forms, green tea (approximately 20%) and black tea (approximately 80%). Extensive studies *(3–12)* from this and other laboratories over the last 10 years have verified cancer chemopreventive effects of a polyphenol mixture derived from green tea against many animal tumor bioassay systems. In these studies, oral consumption or topical application of green tea polyphenols or its major constituent epigallocatechin-3-gallate has been shown to offer protection against all stages of multistage carcinogenesis that include initiation, promotion, and progression. A study *(13)* has also shown that green tea consumption can inhibit the growth of established skin papillomas in mice. Epidemiologic studies have not provided conclusive results but tend to suggest that green tea may reduce the risk associated with cancers of the bladder *(14)*, prostate

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See "Notes" following "References."

(15), esophagus *(16)*, and stomach *(17,18)*. Deciphering the molecular mechanism, by which green tea imparts the protective effects, is important because it may provide opportunities to interfere with cancer development through administration of purified polyphenolic derivatives. Green tea appears to be potentially an ideal agent for chemoprevention. In our judgment, an ideal agent should have the following characteristics: (*a*) little or no adverse effects, (*b*) high efficacy against multiple sites, (*c*) effectiveness at achievable dose levels, (*d*) capability for oral consumption, (*e*) a known mechanism of action, (*f*) low cost, (*g*) history of use by the human population, and (*h*) general human acceptance.

Cancer chemopreventive agents may alter the regulation of cell cycle [*(19)* and references therein]. Treatment with such agents may result in cell cycle arrest, thereby reducing the growth and proliferation of cancerous cells, and may also affect the malignant transformation. It has been suggested that tumor growth depends on evasion of normal control mechanisms that operate through a programmed deletion of cells [i.e., apoptosis *(20)*]. This, in fact, is a physiologic and pathologic process and functions as an essential mechanism of tissue homeostasis. In recent years, many cancer chemopreventive agents have been shown to induce apoptosis *(21–24)* and conversely several tumor promoters have also been shown to inhibit apoptosis *(25)*. We designed this study to investigate if green tea polyphenols and the major constituent, epigallocatechin-3-gallate, induce apoptosis and perturb cell cycle progression in carcinoma cells.

Materials and Methods

Materials

A polyphenolic fraction was prepared from green tea (hereafter referred to as green tea polyphenols) as per the method standardized in our laboratory *(3)*. Briefly, dried green leaves (100 g) were extracted twice with hot water (80 °C) and three times with 80% ethanol (700 mL each time) under nitrogen. The combined extract (3.5 L) was concentrated under vacuum to 1 L and then extracted with an equal volume of chloroform. The aqueous layer was extracted three times with ethyl acetate (800 mL each time) under nitrogen, and the total organic soluble fraction (2.4 L) was concentrated under vacuum. The residue obtained was dissolved in water (50 mL) and freeze-dried. The light-brown solid matter obtained was called green tea polyphenols. Chromatographic analysis of this mixture showed that it contains four major polyphenolic compounds (i.e., epigallocatechin-3-gallate, epicatechin-3-gallate, epigallocatechin, and epicatechin) *(3)*. Purified preparations of all of these polyphenolic compounds (>97% purity) were provided by D. A. Balentine (Lipton Tea Co., Englewood Cliffs, NJ). The cells, namely, human epidermoid carcinoma A431, human carcinoma keratinocyte HaCaT, human prostate carcinoma DU145, and mouse lymphoma L5178Y, were obtained from American Type Culture Collection (Rockville, MD). Normal human epidermal keratinocytes (NHEKs) were prepared from the human foreskin using standard procedure *(26)*. A431 and HaCaT cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin (Life Technologies, Inc. [GIBCO BRL], Gaithersburg, MD). DU145 cells were maintained in RPMI-1640 medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin. L5178Y cells were maintained in Fisher's medium supplemented with 10% equine serum and briefly (3 minutes) gassed with CO_2. NHEK cells were maintained in keratinocyte–serum-free medium (Life Technologies, Inc.) supplemented with L-glutamine, epidermal growth factor, and bovine pituitary extract. The cells were maintained at 37 °C and 5% CO_2 in a humid environment. Green tea polyphenols and all its individual compounds were dissolved in double distilled water for the treatment.

Detection of DNA Fragmentation Induced by Green Tea Polyphenols and Its Individual Constituents

The A431 cells were grown to about 70% confluency and treated with green tea polyphenols (40, 80, and 160 μg/mL) or its individual constituents (40 μg/mL) for 48 hours. In another study, A431, HaCaT, DU145, and L5178Y cells were treated with 80 μg/mL epigallocatechin-3-gallate for 48 hours, whereas NHEK cells were treated with 40, 80, and 160 μg/mL of epigallocatechin-3-gallate for 48 hours. Following these treatments, the cells were washed twice with phosphate-buffered saline (10 m*M* PBS, pH 7.2), suspended in 1 mL cytoplasm extraction buffer (10 m*M* Tris, pH 7.5, 150 m*M* NaCl, 5 m*M* $MgCl_2$, and 0.5% Triton X-100), left on ice for 15 minutes, and pelleted by centrifugation (14 000*g*) at 4 °C. The pellet was incubated with DNA lysis buffer (10 m*M* Tris, pH 7.5, 400 m*M* NaCl, 1 m*M* EDTA, and 1% Triton X-100) for 20 minutes on ice and then centrifuged at 14 000*g* at 4 °C. The supernatant obtained was incubated overnight with RNase (0.2 mg/mL) at room temperature and then with Proteinase K (0.1 mg/mL) for 2 hours at 37 °C. DNA was then extracted using phenol: chloroform (1:1) and precipitated with 95% ethanol for 2 hours at −80 °C. The DNA precipitate was centrifuged at 14 000*g* at 4 °C for 15 minutes and the pellet was air-dried and dissolved in 20 mL of Tris–EDTA buffer (10 m*M* Tris–HCl, pH 8.0, and 1 m*M* EDTA). Total amount of DNA was resolved over 1.5% agarose gel, containing 0.3 μg/mL ethidium bromide in 1× TBE buffer (pH 8.3; 89 m*M* Tris, 89 m*M* Boric acid, and 2 m*M* EDTA) (BioWhittaker, Inc., Walkersville, MD). The bands were visualized under UV transilluminator (Model #TM-36, UVP Inc., San Gabriel, CA) followed by polaroid photography (MP-4 Photographic System, Fotodyne Inc., Hartland, WI).

Detection of Apoptosis by Confocal Microscopy

A431 cells were cultured over round glass coverslips in a 60-mm culture dish to about 70% confluency and then treated with epigallocatechin-3-gallate (20-, 40-, and 80-μg/mL doses) for 48 hours followed by incubation with 10 μ*M* SYTO 13 (Molecular Probes, Inc., Eugene, OR) for 20 minutes in complete medium at 37 °C for labeling the nuclei. The coverslips were washed with PBS and mounted. The SYTO 13 fluorescence was excited by the argon–krypton laser with 488-nm wavelength and imaged through a 460-nm dichroic reflector and a 514–540-nm emission filter with a Zeiss 410 laser scanning confocal microscope. Apoptosis was characterized by the morphologic changes, viz., chromatin condensation, nuclear condensation, and formation of apoptotic bodies.

Quantification of Apoptosis by Flow Cytometry

The A431 cells were grown at a density of 1×10^6 cells in 100-mm culture dishes and were treated with epigallocatechin-3-gallate (20-, 40-, 80-, and 160-μg/mL doses) for 48 hours. The cells were trypsinized, washed with PBS, and processed for labeling with fluorescein-tagged deoxyuridine triphosphate nucleotide and propidium iodide by use of an APO–DIRECT apoptosis kit obtained from Phoenix Flow Systems (San Diego, CA), as per the manufacturer's protocol. The labeled cells were then analyzed by flow cytometry.

Cell Viability

The A431 cells were grown to 70% confluency and treated with epigallocatechin-3-gallate (20-, 40-, 60-, and 80-μg/mL doses) for 48 hours and the cell viability was determined by trypan blue exclusion assay.

DNA Cell Cycle Analysis

The A431 cells (70% confluent) were serum starved for 36 hours to arrest them in G_0 phase of the cell cycle, after which they were treated with epigallocatechin-3-gallate (40 or 80 μg/mL) in DMEM complete medium for 24 hours. The cells were trypsinized thereafter, washed twice with cold PBS, and centrifuged. The pellet was resuspended in 50 μL cold PBS and 450 μL cold methanol for 1 hour at 4 °C. The cells were centrifuged at 1100 rpm for 5 minutes, pellet washed twice with cold PBS, suspended in 500 μL PBS, and incubated with 5 μL RNase (20 μg/mL final concentration) for 30 minutes. The cells were chilled over ice for 10 minutes and stained with propidium iodide (50 μg/mL final concentration) for 1 hour and analyzed by flow cytometry.

Results

We studied whether the green tea polyphenols and the polyphenolic epicatechin derivatives present in it induce apoptosis in human carcinoma cells. As evident by the formation of internucleosomal DNA fragments (Fig. 1, A) compared with ve-

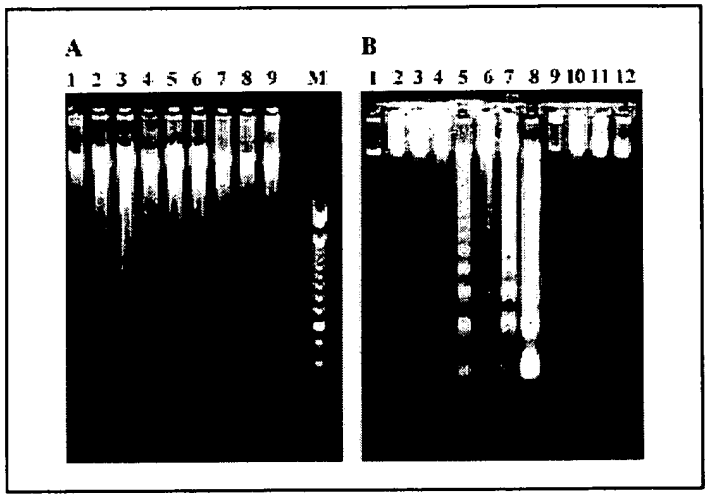


Fig. 1. DNA fragmentation by green tea polyphenols and the individual polyphenolic constituents present therein in different cell types. Cells were treated with vehicle, green tea polyphenols or its individual constituents. Forty-eight hours later, the cells were collected and cellular DNA was isolated and subjected to agarose gel electrophoresis followed by visualization of bands as described in the "Materials and Methods" section. Data shown here are from a representative experiment repeated three times with similar results. **A)** DNA fragmentation in A431 cells. Lane 1, vehicle only; lane 2, green tea polyphenols (40 μg/mL); lane 3, green tea polyphenols (80 μg/mL); lane 4, green tea polyphenols (160 μg/mL); lane 5, epigallocatechin-3-gallate (40 μg/mL); lane 6, epigallocatechin (40 μg/mL); lane 7, epicatechin-3-gallate (40 μg/mL); lane 8, epicatechin (40 μg/mL); lane 9, caffeine (40 μg/mL); and M, molecular weight marker. **B)** DNA fragmentation in different cell types. Lanes 1–4—A431, HaCaT, L5178Y, and DU145 cells, respectively, treated with vehicle alone; lanes 5–8—A431, HaCaT, L5178Y, and DU145 cells, respectively, treated with epigallocatechin-3-gallate (80 μg/mL); lane 9—NHEK treated with vehicle alone; and lanes 10–12—NHEK treated with a 40-, 80-, and 160-μg/mL dose of epigallocatechin-3-gallate, respectively.

hicle-treated control (Fig. 1, A, lane 1), the green tea polyphenols treatment of A431 cells resulted in an induction of apoptosis at 40-, 80-, and 160-μg/mL doses (Fig. 1, A, lanes 2–4, respectively). Among the individual constituents present in green tea polyphenols, epigallocatechin-3-gallate, epicatechin-3-gallate, and epigallocatechin resulted in the induction of apoptosis in A431 cells at a dose of 40 μg/mL, whereas epicatechin and caffeine did not show such effect (Fig. 1, A).

Since epigallocatechin-3-gallate is the major constituent present in green tea polyphenols (approximately 50%, wt/wt), we performed all of the subsequent experiments with epigallocatechin-3-gallate. We next examined if this induction of apoptosis by epigallocatechin-3-gallate is specific for A431 cells or if the other tumor and normal cell lines originating from different body sites also undergo apoptotic cell death following epigallocatechin-3-gallate treatment. As shown in Fig. 1, B, compared with vehicle-treated controls, epigallocatechin-3-gallate (80 μg/mL for 48 hours) treatment of the cells in monolayer or suspension resulted in the formation of DNA fragments in human carcinoma keratinocyte HaCaT (lane 2 versus lane 6), mouse lymphoma cells L5178Y (lane 3 versus lane 7), and human prostate carcinoma cells DU145 (lane 4 versus lane 8). Interestingly, it was important to observe that epigallocatechin-3-gallate did not result in the formation of DNA ladder in NHEK, even at twice the dose (160 μg/mL) of that used for carcinoma cell lines (Fig. 1, B, lanes 9–12).

The induction of apoptosis by epigallocatechin-3-gallate was also evident from the morphologic alterations as shown by confocal microscopy after labeling the cells with SYTO 13 (Fig. 2). Since this method can identify early apoptotic cells, we included the lower concentration of epigallocatechin-3-gallate (20 μg/mL) to assess the possibility of an early apoptotic response. The vehicle-treated control (Fig. 2, A) as well as the low dose (20 μg/mL) of epigallocatechin-3-gallate (Fig. 2, B) did not cause any alteration in the nuclear morphology of A431 cells. At the 40-μg/mL dose of epigallocatechin-3-gallate, several cells displayed early apoptotic morphology, such as peripheral aggregation of nuclear chromatin (Fig. 2, C). However, the plasma

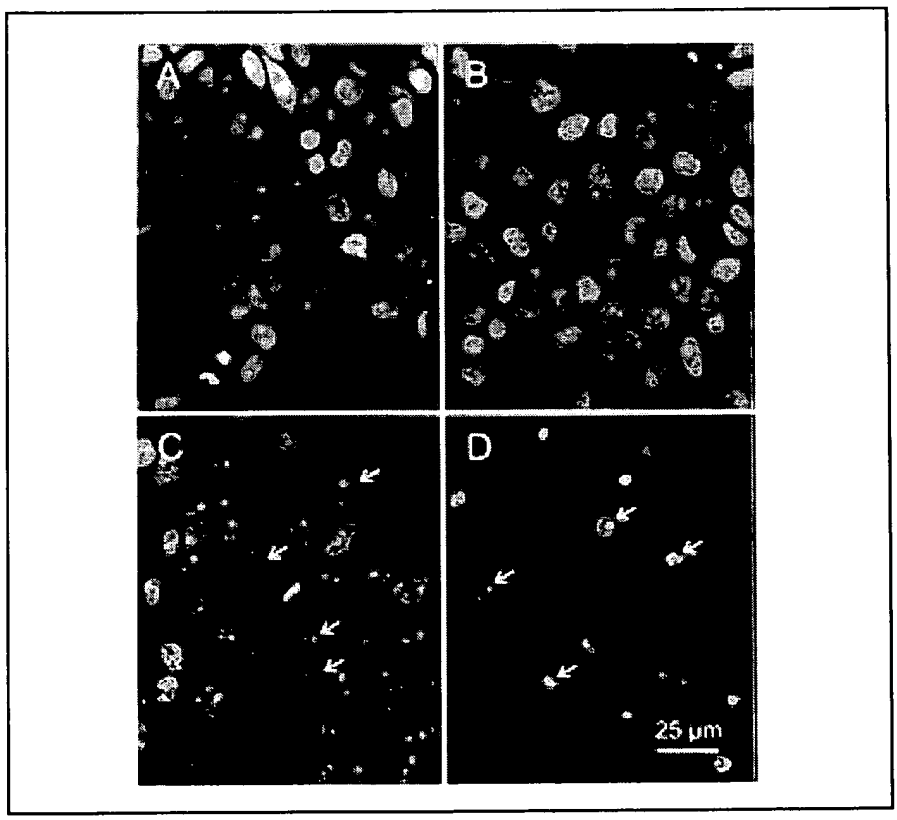


Fig. 2. Morphologic changes in A431 cells following epigallocatechin-3-gallate treatment as evident by confocal microscopy. **A)** vehicle only; **B–D)**, 20-, 40-, and 80-μg/mL dose of epigallocatechin-3-gallate, respectively, for 48 hours. Morphologic changes denoting apoptotic cells are shown by arrows. Bar represents 25 μm. Data shown here are from a representative experiment repeated four times with similar results.

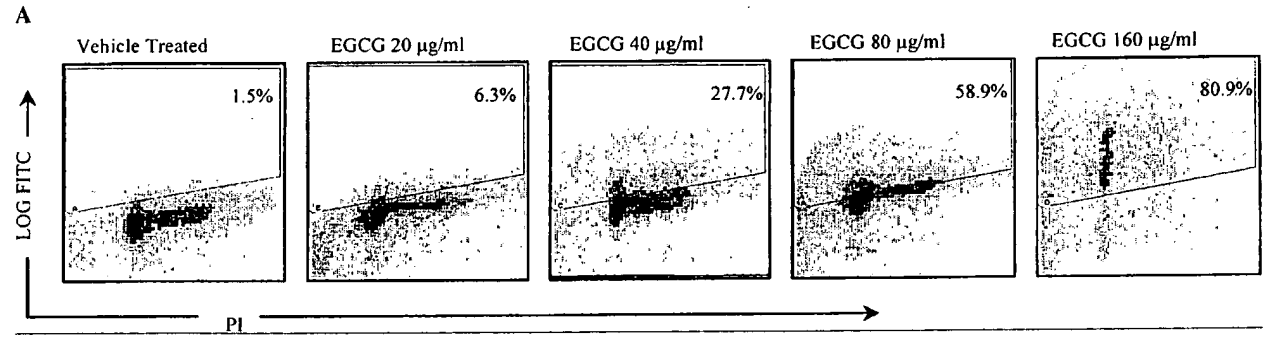
membrane in the epigallocatechin-3-gallate-treated cells did not take up propidium iodide showing that the plasma membrane was still intact. At the highest dose of epigallocatechin-3-gallate (80 μg/mL) that could be tested in this experiment, nearly all cells were found to be in the late stage of apoptosis as evident from advanced chromatin condensation, nuclear condensation, and formation of apoptotic bodies (Fig. 2, D). In addition, the number of cells that could be observed on the coverslip was markedly diminished at 40- and 80-μg/mL doses of epigallocatechin-3-gallate, indicating that the treatment has also resulted in a detachment of the apoptotic cells. We could not assess the extent of apoptosis at 160-μg/mL dose of epigallocatechin-3-gallate because this dose resulted in a total detachment of the cells from the coverslip.

We next quantified the extent of apoptosis by flow cytometric analysis of the cells labeled with deoxyuridine triphosphate and propidium iodide. Since this is a quantitative procedure to measure the extent of apoptosis, for this experiment we selected epigallocatechin-3-gallate treatment at doses of 20, 40, 80, and 160 μg/mL for 48 hours. Such treatments resulted in 6.3%, 27.7%, 58.9%, and 80.9% of apoptotic cells, respectively (Fig. 3, A). While the induction of apoptosis was almost negligible at the lowest dose (20 μg/mL), the highest dose (160 μg/mL) resulted in a massive apoptosis and a drastic decline in number of cells as determined by flow cytometry. When we assessed the effect of epigallocatechin-3-gallate on the viability of A431 cells by dye-exclusion technique, there was no loss in cell viability at the 20-μg/mL dose but a considerable dose-dependent loss of viability was observed at concentrations of 40, 60, and 80 μg/mL (Fig. 3, B).

Since the induction of apoptosis may be mediated through the regulation of cell cycle, we also examined the effect of epigallocatechin-3-gallate on cell cycle perturbations. Compared with vehicle-treated controls, the epigallocatechin-3-gallate treatment resulted in an appreciable arrest of A431 cells in G_0–G_1 phase of the cell cycle after 24 hours of the treatment. The treatment caused an arrest of 51% cells in G_0–G_1 phase of the cell cycle at a 40-μg/mL dose, which further increased to 74% at the higher dose of 80 μg/mL (Fig. 4), which did not change further at 160 μg/mL (data not shown). This increase in G_0–G_1 cell population was accompanied with a decrease of cell number in S phase. However, the G_2–M cell population remained essentially unchanged. At earlier time points and at a low dose of 20 μg/mL, there was no change in distribution of cells in the cell cycle following treatment with epigallocatechin-3-gallate (data not shown).

Discussion

As a part of our ongoing large-scale program on cancer chemoprevention through dietary constituents, the main aim of this study was to elucidate the mechanism of antitumorigenic effect of epigallocatechin-3-gallate, the major polyphenolic agent present in green tea, and to determine if it directly affects the cell cycle regulation and apoptosis. Apoptosis, in recent years, has become an important issue in biomedical research. The life spans of both normal and cancer cells within a living system are regarded to be substantially affected by the rate of apoptosis. In addition, apoptosis is a discrete



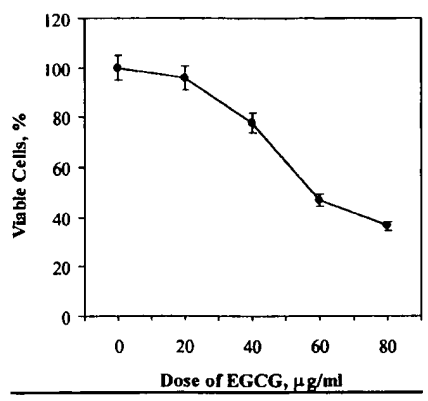


Fig. 3. A) Apoptosis by flow cytometry. A431 cells were treated with epigallocatechin-3-gallate (20, 40, 80, and 160 μg/mL for 48 hours), labeled with deoxyuridine triphosphate using terminal deoxynucleotide transferase and propidium iodide and analyzed by flow cytometry. Results are displayed using the dual-parameter method where the *X* and *Y* axes represent DNA and deoxyuridine triphosphate fluorescence, respectively. Cells showing deoxyuridine triphosphate fluorescence above that of the control population, as indicated by the line in each histogram, are considered as apoptotic cells and their percent population is shown at the right top in each box. Data shown here are from a representative experiment repeated three times with similar results. **B)** Viability of the A431 cells following epigallocatechin-3-gallate treatment. A431 cells were incubated with epigallocatechin-3-gallate (20, 40, 60, and 80 μg/mL for 48 hours) and the number of viable cells was counted by trypan blue exclusion assay. Cell viability is represented as percent viable cells, where the vehicle-treated cells are regarded as 100%. Data shown are mean values ± standard errors determined from three independent experiments.

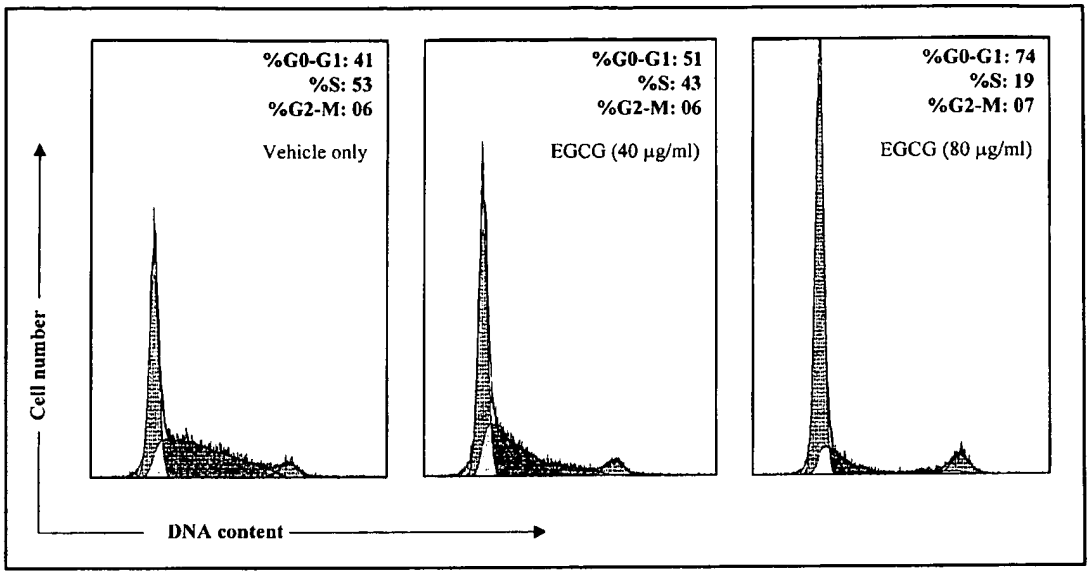


Fig. 4. DNA flow cytometric analysis. A431cells were treated with vehicle or epigallocatechin-3-gallate (40 and 80 μg/mL) for 24 hours and analyzed by flow cytometry. Percentages of cells in G_0–G_1, S, and G_2–M phase were calculated using Cellfit computer software and are represented within the histograms. Data shown here are from a representative experiment repeated two times with similar results.

way of cell death different from necrotic cell death and regarded to be an ideal way of cell elimination. Thus, the chemopreventive agents, which can modulate apoptosis, may be able to affect the steady-state cell population that are often useful targets in the management and therapy for cancer.

In this study, we have shown that green tea polyphenols and their constituents, viz., epigallocatechin, epicatechin-3-gallate, and epigallocatechin-3-gallate resulted in an induction of apoptosis in A431 cells. Epigallocatechin-3-gallate possesses two triphenolic groups in its structure, which are reported to be important for its stronger biologic activity *(27)*. We selected this polyphenol for the detailed study because it is the major polyphenol of the green tea polyphenols and is primarily responsible for the green tea effect. It is important to note that epigallocatechin-3-gallate resulted in the induction of apoptosis in all carcinoma cells but not in the normal cells we examined. This selectivity, if it can be observed *in vivo* at the desirable doses, will be of great therapeutic importance. A vast variety of the chemotherapeutic agents, currently used in cancer therapy, are thought to kill the cells by the mechanisms other than apoptosis. This may not always be a preferable way of cancer management. Our results showing the induction of apoptosis by epigallocatechin-3-gallate is of importance because epigallocatechin-3-gallate treatment of A431 cells did not cause any necrosis as evident from the confocal microscopy. The cells were stained with SYTO 13 that is indicative only of apoptosis. The cells did not take up propidium iodide, which also stains the necrotic cells. Moreover, the four different carcinoma cells used in our study have origins from different body sites. This showed that epigallocatechin-3-gallate may be useful against many, if not all, types of cancers. The demonstration of induction of apoptosis by epigallocatechin-3-gallate is also important because green tea is a well-consumed beverage worldwide and has shown promising cancer chemopreventive effects in both laboratory experiments and human epidemiologic studies.

The effect of epigallocatechin-3-gallate in arresting the A431 cells in G_0–G_1 phase of the cell cycle suggests the possibility that green tea may also be useful for the control of cancer growth. Most of the cancer types known to date have defects in one or more cell cycle checkpoints. For example, a number of studies *(28–30)* have shown an implication of p53-mediated induction of WAF1/Cip1/p21, resulting in a G_1 cell cycle arrest. The loss of cell cycle checkpoints results in the selection of cells that have a growth advantage and a predisposition for acquiring more chromosome aberrations. This may also result in drug resistance, invasion, and metastasis. The G_0–G_1 arrest shown by epigallocatechin-3-gallate, therefore, suggests that this agent may slow down the growth of cancer cells by artificially imposing the cell cycle checkpoint. The exact mechanism(s) of apoptosis and cell cycle deregulation by epigallocatechin-3-gallate needs a further exploration of genetic and signal transduction pathways. It is, however, tempting to speculate that cyclin kinase inhibitor(s), cyclin dependent kinase(s) and their regulatory cyclin(s) proteins operating in G_1 phase of the cell cycle may be involved in the epigallocatechin-3-gallate-mediated apoptosis and cell cycle arrest. The involvement of more than one independent or interdependent pathways for apoptotic and cell cycle deregulatory response of epigallocatechin-3-gallate is a possibility that needs to be further explored. In summary, based on our findings reported here and on the extensive amount of laboratory and epidemiology data available, we suggest that clinical trials with epigallocatechin-3-gallate are needed in a population with high cancer risk.

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Notes

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